PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 09 January 2001 (09.01.01)	
International application No. PCT/US00/06862	**Applicant's or agent's file reference** 58857-A-PCT
International filing date (day/month/year) 15 March 2000 (15.03.00)	**Priority date** (day/month/year) 15 March 1999 (15.03.99)
Applicant FISHER, Paul, B. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

10 October 2000 (10.10.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

US0006862

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 58857-A-PCT	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US00/06862	International filing date *(day/month/year)* 15 MARCH 2000	(Earliest) Priority Date *(day/month/year)* 15 MARCH 1999
Applicant THE TRUSTEE OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
   [ ] contained in the international application in written form.
   [ ] filed together with the international application in computer readable form.
   [ ] furnished subsequently to this Authority in written form.
   [ ] furnished subsequently to this Authority in computer readable form.
   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
2. [ ] **Certain claims were found unsearchable** (See Box I).
3. [ ] **Unity of invention is lacking** (See Box II).
4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established by this Authority to read as follows:
5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the **drawings** to be published with the abstract is Figure No. _____
   [ ] as suggested by the applicant.
   [ ] because the applicant failed to suggest a figure.
   [ ] because this figure better characterizes the invention.
   [X] None of the figures.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
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CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

NOTES TO FORM PCT/ISA/220

These Notes are intended t give the basic instructions concerning the filing of amendments under Article 19. The Notes are based on the requirements of the Patent Cooperation Treaty and of the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule" and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended ?

The claims only.

The description and the drawings may only be amended during international preliminary examination under Chapter II.

When ? **Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).**

Where not to file the amendments ?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been filed, see below.

How ? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

What documents must/may accompany the amendments ?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confounded with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;
(ii) th claim is cancelled;
(iii) the claim is new;
(iv) the claim replaces one or more claims as filed;
(v) the claim is the result of the division f a claim as filed.

NOTES TO FORM PCT/ISA/220 (continued)

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under Article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

The statement should be brief, it should not exceed 500 words if in English or if translated into English.

It should not be confounded with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It should not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

In what language ?

The amendments must be made in the language in which the international application is published. The letter and any statement accompanying the amendments must be in the same language as the international application if that language is English or French; otherwise, it must be in English or French, at the choice of the applicant.

Consequence if a demand for international preliminary examination has already been filed ?

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase ?

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

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PATENT COOPERATION TREATY

PCT

RECD 24 JUL 2001

WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 58857-A-PCT	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/06862	International filing date *(day/month/year)* 15 MARCH 2000	Priority date *(day/month/year)* 15 MARCH 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK		

1. **This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.**

2. **This REPORT consists of a total of 62 sheets.**

   [X] **This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).**

   These annexes consist of a total of 3 sheets.

3. **This report contains indications relating to the following items:**

   - I [X] **Basis of the report**
   - II [ ] **Priority**
   - III [ ] **Non-establishment of report with regard to novelty, inventive step or industrial applicability**
   - IV [ ] **Lack of unity of invention**
   - V [X] **Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**
   - VI [ ] **Certain documents cited**
   - VII [ ] **Certain defects in the international application**
   - VIII [X] **Certain observations on the international application**

Date of submission of the demand	Date of completion of this report
10 OCTOBER 2000	29 MAY 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer SHIN-LIN CHEN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)*

INTERNATIONAL PRELIMINARY [redacted]MINATION REPORT

Internat[redacted]l application No.
PCT/US00/06862

I. Basis f the report

1. With regard to the elements of the international application:*

☐ the international application as originally filed

☒ the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the claims:
pages (See Attached) , as originally filed
pages _____ , as amended (together with any statement) under Article 19
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the drawings:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the sequence listing part of the description: (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIM[redacted]RY EXAMINATION REPORT

Inter[redacted]al application No.
PC[redacted]S00/06862

V. Reasoned statement under Article 35(2) with regard t novelty, inventive step r industrial applicability; citations and explanati ns supporting such statement

1. **statement**

Novelty (N)	**Claims**	1-20	**YES**
	Claims	NONE	**NO**
Inventive Step (IS)	**Claims**	NONE	**YES**
	Claims	1-20	**NO**
Industrial Applicability (IA)	**Claims**	1-20	**YES**
	Claims	NONE	**NO**

2. **citations and explanations (Rule 70.7)**

Claims 1-8, 11-17 , 19 and 20 lack an inventive step under PCT Article 33(3) as being obvious over Gossen et al., 1995 (A) in view of Goldman et al., 1996 (B).

Claims 1-8 are directed to a vector comprising a nucleic acid encoding rtTA under the control of a human EF-1 a promoter and a gene of interest under the control of said promoter, and an isolated cell comprising said vector. Claims 11-17 are directed to a method of generating a rtTA expression system for inducible tetracycline regulated gene expression, comprising rtTA DNA and EF-1 a promoter, and a vector generated by said method. Claims 19 and 20 are directed to a method for expressing a gene of interest by contacting the cell as set forth above with an inducer such as tetracycline or deoxycycline to cause the cell to express the gene of interest.

Reference A modified the Tet repressor (TetR) amino acid sequence so that the mutated repressor obtains a reverse properties as compared to wild-type Tet repressor, wherein the reverse TetR will bind to the operator in the presence of tetracycline or its derivatives such as doxycycline, and generated constructs pUHD17-1neo and pUHD172-1neo encoding rtTA or rtTA-nls (nuclear localization signal) under the control of human CMV promoter, respectively. Reference A also established stable Hela cell lines that produced rtTA or rtTA-nls using pUHD17-1neo and pUHD172-1neo, respectively, and demonstrated dramatically increased enzyme activity of beta-galactosidase or luciferase under the control of a rTA-dependent promoter upon the induction of doxycycline (e.g. pages 1767, 1768). Therefore, reference A established a tetracycline inducible rtTA expression system comprising a vector encoding rtTA under the control of human CMV promoter.

Reference A does not teach putting rtTA under the control of EF-1 alpha promoter.

Reference B teaches constructing the plasmid pcDEF3 which is derived from plasmids pEF-Bos and pcDNA3, wherein pcDEF3 contains EF-1 alpha promoter. Reference B shows higher expression levels of human IFNAR-1 using pcDEF3 which contains EF-1 alpha promoter than using pcDNA3 which contains CMV promoter but lacks EF-1 alpha promoter (e.g. page 1014). The recombinant DNA techniques described in claims 11-16 such as sticky end ligation, blunt end ligation and blunting the end by klenow fragment were well known in the art and would be obvious for a ordinary artisan. With the collective teachings of A and B, it would have motivated one of ordinary skill to subclone rtTA DNA fragment into pcDEF3 vector since EF-1 alpha promoter shows stronger activity than human CMV promoter for certain type of protein expression and the availability of tetracycline inducible rtTA expression system.

Applicants argue that there is no motivation to combine the teaching of Gossen (A) with that of Goldman (B), and Gossen does not provide any motivation to use the promoter of Goldman. Applicants further argue that Goldman teaches a simple expression system but Gossen teaches complicated expression system which produces excellent dose-response characteristics and one of ordinary skill would not be motivated to substitute EF-1 alpha promoter for CMV promoter to upset the balance with a different promoter. This is not found persuasive. Gossen established a tetracycline inducible rtTA expression system comprising a vector encoding rtTA under the control of an appropriate promoter, and demonstrated the expression of a b-galactosidase or a luciferase under the control of a CMV promoter or a thymidine (Continued on Supplemental Sheet.)

VIII. Certain bservations n the internati nal application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because:

Claims 9, 10 and 18 encompass any transgenic animal, including mammals, fish, bird, arthropod etc, comprising a vector containing nucleic acids which encodes rtTA under the control of EF-1 alpha promoter.

The specification discloses the construction of plasmid EF1prtTA and the establishment of stable Tc-responsive clonal cell lines in HO-1, MCF-7, PC3 and DU-145 cells expressing the rtTA cDNA under the regulation of the EF-1 alpha promoter. The specification fails to provide an enabling disclosure for the preparation of any and all transgenic animal, because it fails to provide sufficient guidance for the preparation of any transgenic animal and further because it fails to provide a suitable description of resulting phenotypes of the claimed transgenic animals.

The state of the art in the fields of transgenic animal at the time of the invention was unpredictable, the transgene expression and physiological results of such expression is not always accurately predictable (Palmiter et al., 1983 (e.g. abstract); Pursel et al. 1990(e.g. abstract)). The individual gene of interest, promoter, enhancer, coding or non-coding sequences present in the transgene construct, the site of integration, etc., are the important factors that governs the expression of a transgene (Kappel et al., 1992, p. 549). However, the specification fails to provide adequate guidance in regard to what type of vector, coding and non-coding sequence would have been useful in combination with which animal and which target cell type. Therefore, the specification has not enabled the generation and use of a transgenic animal comprising a vector containing nucleic acids which encodes rtTA under the control EF-1 alpha promoter.

Claims 9, 10 and 18 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

Applicants argue that the art has taught the method of making transgenic animals, and test breeding of said animals. this is not found persuasive because of the reasons set forth above and that although the method of making transgenic animals was known in the art, however the resulting phenotypes of a transgenic animal was unpredictable at the time of the invetnion. Absent of the resulting phenotypes of a transgenic animal, one skilled in the art at the time of the invetnion would not know how to use said transgenic animal.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C12N 15/00, 5/00, 15/63; C12P 21/06; C12Q 1/68; A01K 67/00 and US Cl.: 435/6, 320.1, 325, 69.1, 455; 800/13, 18

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
page(s) 1-28, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
page(s) NONE, as originally filed.
page(s) NONE, as amended under Article 19.
page(s) NONE, filed with the demand.
and additional amendments:
Pages 29-31 filed with the letter of 07 May 2001.

This report has been drawn on the basis of the drawings,
page(s) 1-5, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the sequence listing part of the description:
page(s) NONE, as originally filed.
pages(s) NONE, filed with the demand.
and additional amendments:
NONE

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
kinase promoter upon the induction of doxycycline in eukaryotic cells. Goldman teaches substitution of a CMV promoter in a pcDNA3 vector with a human EF-1 alpha promoter dramatically increased cell surface expression levels of the receptor tested in eukaryotic cells (e.g. p. 1014, 1015). Both Gossen and Goldman teaches an expression system for expressing a gene product of a gene of interest in eukaryotic cells. Although Gossen does not teach using a human EF-1 a promoter for the expression of a gene product, Goldman teaches substituting a CMV promoter with a human EF-1 a promoter to increase the expression of a gene product of a gene of interest. Thus, one ordinary skill in the art at the time of the invention would have been motivated to substitute CMV promoter used in the rtTA expression system as taught by Gossen with the human EF-1 a promoter for higher expression of a gene product as taught by Goldman because the human EF-1 a promoter results in higher gene expression of cell surface receptors than CMV promoter does. One ordinary skill in the art would expect to express a gene product with a rtTA expression system under the control of a human EF-1 a promoter because Gossen teaches the expression of a b-galactosidase or a luciferase using the rtTA expression system.

Applicants argue that Goldman "admit having problems with transient and stable expression of both aforementioned subunits of the human Type I interferon receptor (IFNAR-1 and IFNAR-2) as well as the human Type II interferon receptor subunit IFNGR-1 in pcDNA3". This is not found persuasive because the problems with transient and stable expression set forth above are irrelevant to the expression system taught by Gossen which does not use pcDNA1 expression vector and said expression system has been shown to be successful.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII **Sheet 11**

Claims 1-20 lack an inventive step under PCT Article 33(3) as being obvious over Bujard et al., 1997 (C) in view of Goldman et al., 1996 (B).

Claims 1-8, 11-17, 19 and 20 are as discussed above. Claims 9 and 10 encompass any transgenic non-human animal, including mammals, fish bird, arthropod etc, comprising a vector containing nucleic acids which encodes rtTA under the control of EF-1 a promoter. Claim 18 is directed to a method for screening for an anti-tumor drug coprising administering to a transgenic non-human animal a drug to induce or repress expression of a gene of interest under regulation of tetracycline or deoxycycline and said gene of interest is associated with cancer.

Reference C teaches constructing plasmid pUHD17-1neo and pUHD172-1neo encoding rtTA or rtTA-nls (nuclear localization signal) under the control of human CMV promoter, respectively. Reference C teaches establishing stable Hela cell lines that produce rtTA or rtTA-nls using pUHD17-1neo and pUHD172-1neo, respectively, demonstrated dramatically increased enzyme activity of beta-galactosidase or luciferase under the control of a rTA-dependent promoter upon the induction of doxycycline, and suggest transgenic or homologous recombinant animals which comprise both nucleic acid components of the expression system can be created by introducing both nucleic acids into same cells at an embryonic stage. Reference C also teaches a method for stimulating transcription of the nucleotide sequence operatively linked to the tet operator sequence in the transfected host cell by contacting the host cell with tetracycline or a tetracycline analogue, and a kit that can be used to regulate the expression of a gene of interest (e.g. columns 20, 25, 33, 34, 49, 50).

Reference C does not teach putting rtTA under the control of EF-1 alpha promoter.

Reference B teaches constructing the plasmid pcDEF3 which is derived from plasmids pEF-Bos and pcDNA3, wherein pcDEF3 contains EF-1 a promoter. B shows higher expression levels of human IFNAR-1 using pcDEF3 which contains EF-1 a promoter than using pcDNA3 which contains CMV promoter but lacks EF-1 alpha promoter (e.g. page 1014). For the same reasons set forth above, it would have been obvious for one of ordinary skill at the time of the invention to practice the claimed invention.

Applicants argue that the choice of promoter and vector of Bujard is different from that of Goldman and there is no motivation to combine the teachings of Goldman and Bujard. Applicants further argue that Bujard teaches a preferred recombinant expression vector and there is no motivation to replace a the CMV promoter with EF-1 alpha promoter. This is not found persuasive. A preferred recombinant vector does not mean that other vector with different promoter should not be used or further tested for better gene expression. Bujard established stable Hela cell lines comprising a tetracycline inducible rtTA expression system under the control of a promoter such as a CMV promoter that produced rtTA or rtTA-nls using pUHD17-1neo and pUHD172-1neo, respectively, and demonstrated the expression of a b-galactosidase or a luciferase upon the induction of doxycycline. Goldman teaches substitution of a CMV promoter in pcDNA3 vector with a human EF-1 a promoter dramatically increased cell surface expression levels of the receptor tested in eukaryotic cells (e.g. p. 1014, 1015). Both Bujard and Goldman teaches an expression system for expressing a gene product of a gene of interest in eukaryotic cells. Although Bujard does not teach using a human EF-1 a promoter for the expression of a gene product, Goldman teaches a human EF-1 a promoter increases the expression of a gene product when compared to using a CMV promoter. Thus, one ordinary skill in the art at the time of the invention would have been motivated to substitute CMV promoter used in the rtTA expression system as taught by Bujard with the human EF-1 a promoter for higher expression of a gene product as taught by Goldman because the human EF-1 a promoter results in higher gene expression of cell surface receptors than CMV promoter does. One ordinary skill in the art would expect to express a gene product of interest with a rtTA expression system under the control of a human EF-1 a promoter because Bujard teaches the expression of a b-galactosidase or a luciferase using the rtTA expression system.

Applicants argue that Bujard and Goldman do not teach or suggest a recombinant expression vector which provides consistent expression in a cell. This is not found persuasive because the expression system taught by Bujard would provide consistent expression in a cell when tetracycline is provided consistently.

---------- NEW CITATIONS ----------

NONE

IMPROVED EXPRESSION VECTOR FOR CONSISTENT CELLULAR EXPRESSION OF THE TET ON REPRESSOR IN MULTIPLE CELL TYPES

This application claims priority and is a continuation-in-part application of U.S. Serial No. 09/268,303, filed March 15, 1999, the contents of which is hereby incorporated by reference.

The invention disclosed herein was made with Government support under Grant No. CA 35675 from the National Institutes of Health, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Background of the Invention

Since the first report by Gossen and Bujard (Gossen and Bujard, 1992) and subsequent documentation of a variant form (Gossen et al., 1995), the Tetracycline (Tc)-regulated system, has been broadly adopted and is widely acknowledged as the method of choice, in experiments requiring inducible expression of genes of interest. In its originally reported form, the system employs two plasmids. One expressing the tTA or rtTA cDNA (henceforth jointly referred to as TA), a fusion protein of the bacterial Tc-repressor, fused to the C-terminal acidic activation domain of the Herpes Simplex virus (HSV), VP16 transcriptional transactivator. The second plasmid enables cloning of a cDNA of interest downstream of a heptamerized Tc-operator transcription regulatory DNA sequence, fused to a DNA element providing basal promoter

activity, derived either from the CMV IE or HSV thymidine kinase promoters. Establishing a cell line having Tc-regulatable expression of the gene of interest involves a two step process. In the first, a cell line stably expressing the TA cDNA is established and identified by clonal selection and expression analysis through transient transfection with a Tc-responsive reporter. In the second step, the gene of interest cloned under control of the Tc-responsive element is introduced into the cell line made in the previous step and a second round of selection is performed to identify clones displaying Tc-responsive inducibility of the cDNA(Gossen and Bujard, 1992; Gossen et al., 1995). The Tc-regulated system has effectively overcome several drawbacks seen in earlier systems which showed high basal levels of expression, poor responsiveness and toxicity of the inducing agent. The Tc-inducible system is in addition, able to achieve induction over ranges of several orders of magnitude in a graded manner, responsive to varying levels of inducer. Furthermore, the system is extremely versatile and amenable to several types of modifications, permitting the study of the role of a particular gene, or combinations thereof, in a wide variety of cell types of interest. The potential to use this system in medical applications including gene therapy protocols and pharmacological small molecule screening are areas of active investigation. Its versatility has enabled adaptation to situations requiring inducible gene expression in a tissue specific or generalized manner in animal or plant models, opening new avenues to study gene function *in vivo*.

The Tc-inducible expression system has been modified in several ways, in attempts to improve performance or tailor it to specific needs. Autoregulatory control was achieved by placing both the tTA as well as exogenous cDNA under control of Tc-operator sequences (Shocket et al., 1995), which reportedly permitted regulation of available tTA levels only

on induction and thereby increased overall performance in terms of inducibility and frequency of positive clones obtained. Single plasmid vectors containing the tTA sequence and gene of interest in opposite orientations have been developed to obviate the need for multiple rounds of clonal selection(Baron et al., 1995; Schultze et al., 1996; Weinmann et al., 1994). Overcoming a sometimes considerable barrier of introduction of DNA into transfection recalcitrant cells has been made possible through the development of retroviral vectors for delivery of both components of the system in either a single or combination of two separate viruses (Bohl et al., 1997; Hofmann et al., 1996; Kringstein et al., 1998; Paulus et al., 1996; Rossi et al., 1998). Several promoters have been used to enable generalized or tissue specific expression of tTA in plants (Weinmann et al., 1994) or animals (Efrat et al., 1995; Fishman et al., 1994; Furth et al., 1994; Hennighausen et al., 1995). Modification of the Tc-operator containing plasmid to reduce leaky expression or reduce the effects of integration site has been attempted. Strategies toward this end include Epstein Barr virus (EBV) replication origin based vectors that are maintained episomally (Jost et al., 1997), modified basal promoters to reduce uninduced expression (Hoffmann et al., 1997) and incorporation of sequences that prevent interference from adjoining elements at the site of integration (Hennighausen et al., 1995; McKnight et al., 1992; Stief et al., 1989).

The original report and several other studies have documented potential pitfalls and have provided troubleshooting strategies using the Tc regulated system (reviewed in Blau and Rossi, 1999; Gossen et al., 1994; Shockett and Schatz, 1996)). However, anecdotal evidence non-rigorously documenting failure to establish cell lines that show any significant levels of expression or inducibility of the exogenously introduced gene (Ackland-Berglund and Leib, 1995; Gossen and Bujard, 1995) exists. Drawing upon previous

experiences using expression constructs with strong viral promoters based on CMV or SV-40 derived sequences, extinction of expression of transactivator function could be a potentially significant factor encountered in the inability to establish Tc-responsive cell lines. This might be of special relevance in cells having a relatively slow growth rate and/or the potential to differentiate, making them particularly sensitive to this phenomenon, since changes in cell physiology could affect the activity of exogenously introduced viral promoter constructs. The time lapsed between establishing the initial TA expressing clone and identification of cell lines inducibly expressing the gene of interest, is of a sufficient duration, during which the host cell possibly stops supporting CMV promoter enhancer expression, resulting in the shutdown of TA expression. Despite the recent introduction of retroviral vectors that enable single step and therefore relatively quick selection of positive clones, several of these also depend on viral promoters for expression of one or more elements and are therefore also prone to similar problems. The construction of a specific retrovirus is in itself time consuming and a not as yet routine procedure in many laboratories, compared to transfection or electroporation of plasmid DNA into cells. Based on these factors modification of the existing construct for rtTA cDNA expression was done by placing it under the regulation of the human Protein Translation Peptide Elongation Factor-1 α promoter (EF-1 α). This gene has a housekeeping function in all cells and has been documented to be expressed to relatively high levels. More importantly, due to its indispensable housekeeping function in all cells, Protein Translation Peptide Elongation Factor-1 α promoter (EF-1 α) expression is consistent from a temporal viewpoint, relatively insulated from changes in cell physiology and is cell type independent (Goldman et al., 1996; Kim et al., 1990; Wakabayashi-Ito and Nagata, 1994). Utilization of this construct in cells lines derived from diverse human tissues

enabled the successful construction of Tc-regulatable lines in every case attempted so far. This modified vector will not only be of general utility but will be especially useful in cases where difficulties have been previously experienced in successfully establishing Tc-responsive clones.

Summary of the Invention

The present invention provides a cell comprising the vector set forth above. The present invention further provides that the cell is from a cell line. The present invention further provides that the cell line is HeLa (human cervix), HO-1 (human melanoma), MCF-7(human breast), PC3 (human prostate) or DU-145 (human prostate).

The invention also provides an animal comprising the vector set forth above. This invention also provides an animal which comprises a cell which comprises Protein Translation Peptide Elongation Factor-1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor-1 α promoter. This invention also provides the animal includes but is not limited to a mouse.

The present invention provides a method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression comprising: (a)isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion (b)generation of Protein Translation Peptide Elongation Factor-1 α promoter vector, by restriction enzyme digestion (c)directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor -1 α promoter vector by ligation of 5' EcoRI compatible restriction enzyme overhangs (d)directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor -1 α promoter vector by Klenow fragment mediated blunt end generation of 3' Bam HI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3' Xbal end of Protein Translation Peptide Elongation Factor -1 α promoter vector and (e)blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor -1 α promoter vector expressing reverse tetracycline

controlled transactivator.

This invention provides the fragment includes but is not limited to an *Eco* RI-*BAM* HI fragment, the mammalian expression vector includes but is not limited to pCDEF3, cloning is at the 5' *Eco* RI and 3' *BAM* HI of the insert and the ligation is at the 5' *Eco* RI site and the 3'*Xba*l site of pCDEF3.

This invention provides a method of screening pharmacological products using the vector. Finally, this invention provides a method for monitering inducible gene expression in a tissue specific of generalized manner using the vector.

Brief Description of the Figures

Figure 1

Plasmid map of Protein Translation Peptide Elongation Factor-1 α expression construct: The map shows individual component elements of the vector including the rtTA ORF, human EF-1 α promoter, Bovine growth hormone (BGH) polyadenylation (poly A) signal and partial multiple cloning site retained from the vector pCDEF3(Goldman et al., 1996) after cloning. The Neomycin resistance marker (NeoR) flanked by the SV40 promoter and poly A signal, Ampicillin resistance marker (AmpR) for bacterial propagation and selection and some reference restriction site are also shown.

Figure 2

Luciferase assay to test activity of the Protein Translation Peptide Elongation Factor -1 α promoter vector: Extracts from human HO-1 melanoma cells transiently co-transfected with the original (bars marked pUHD 17-1neo) or modified (bars marked EF1p Tet on) rtTA expression vectors and the Tc luciferase reporter pUHC 13-3 were quantitated for luciferase activity. These extracts were prepared from cells treated without the inducer (-Dox) or with (+Dox). Treatment with inducer was for 48h as described in materials and methods.

Figure 3

Luciferase assay to select Tc-inducible clones: Panels show quantitation of luciferase assays from individual Neomycin resistant clonally isolated cell lines of human prostate (DU-145 and PC3), cervical (HeLa), breast (MCF-7) and melanoma (HO-1) tumor origin. Each stable clone was transiently transfected with the Tc luciferase reporter pUHC 13-3 in the absence (-Dox) or presence (+Dox) of inducer. Extracts prepared from these cells were assayed

for luciferase activity to identify clones showing adequate levels of inducibility for each cell type as described in material and methods.

Figure 4

Northern blot analysis of individual Tc responsive clones expressing regulatable Mda-7 or Jun B cDNAs: Autoradiographic detection of levels of induced RNA message levels expressed in clonally selected cells stably transfected with the Mda-7 (A) or Jun B (B) cDNAs under regulation of Tc, probed with respective radiolabelled cDNA probes after transfer to nylon membranes. Each similarly numbered sample was derived from the same clone without induction [1-17(A) and 1-9 (B) or after addition of inducer, 1'-17'(A) and 1'-9' (B)].

Figure 5

Plasmid name: pEF1ptTA. **Plasmid size:** 7.02kb. **Constructed by:** Gopalkrishnan et al., Nucleic Acids Research 27: 4775-4782 (1999) & refs. therein. **Construction date:** January 2000. **Comments/References:** The ORF for the tetracycline repressor VP16AAD fusion driven by Human EF-4 alpha promoter. Source of tTA was pUHD15-1, isolated as an EcoRI/BamHI fragment and cloned into the 5' EcoRI-BamHI (blunted) 3' Xbal (blunted) site of pCDEF3.

Detailed Description of the Invention

The present invention provides a vector comprising an Protein Translation Peptide Elongation Factor -1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor -1 α promoter. In an embodiment the vector is a plasmid. In another embodiment the vector is as set forth in figure 1.

The present invention further provides a cell comprising the vector set forth above. In an embodiment the cell is from a cell line. In a further embodiment the cell line is HeLa (human cervix), HO-1 (human melanoma), MCF-7(human breast), PC3 (human prostate) or DU-145 (human prostate).

The invention also provides an animal comprising the vector set forth above. An embodiment of this invention the vector has been introduced into the animal or an ancestor of the animal at an embryonic stage. The animal includes but is not limited to a mouse.

This invention also provides an animal which comprises a cell which comprises Protein Translation Peptide Elongation Factor-1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor -1 α promoter.

The present invention provides a method of generating a A method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression comprising: (a)isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion(b)generation of Protein Translation Peptide Elongation Factor -1 α promoter vector, by restriction enzyme digestion (c)directional cloning of reverse tetracycline controlled

transactivator into Protein Translation Peptide Elongation Factor -1 α promoter vector by ligation of 5' EcoRI compatible restriction enzyme overhangs (d)directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor -1 α promoter vector by Klenow fragment mediated blunt end generation of 3' Bam HI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3' XbaI end of Protein Translation Peptide Elongation Factor -1 α promoter vector and (e)blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor -1 α promoter vector expressing reverse tetracycline controlled transactivator.

In accordance with the method of the invention, the fragment includes but is not limited to an *Eco* RI-*BAM* HI fragment, the mammalian expression vector includes but is not limited to pCDEF3, cloning is at the 5' *Eco* RI and 3' *BAM* HI of the inserts and the ligation is at the 5' *Eco* RI site and the 3'*Xba*l site of pCDEF3.

The present invention provides a vector which is directed to providing a consistent cellular expression of the tetracycline repressor in cells. Such a vector may be useful in situations requiring inducible gene expression in a tissue specific or generalized manner in animal or plant models. In one embodiment of the invention, pharmacological products are monitored to determine use in medical applications. In the preferred embodiment monitoring is of the gene changes associated with cellular process such as aging, cancer, development, differentiation and growth.

More specifically, methods which are well known to those skilled in the art can be used to construct a vector directed to providing a cellular expression of the tetracycline repressor in cells. These methods include in cell culture

techniques, northern blotting, enzyme activity analysis, construction of plasmids and sequencing. See e.g., the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

As used herein the term "tetracycline controlled transactivator" encompasses a vector expressing a protein that binds and activates transcription of downstream tetracycline induced operator binding elements, only when tetracycline is present.

This invention provides a method of screening pharmacological products using the vector. Finally, this invention provides a method for monitering inducible gene expression using the vector.

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in understanding the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Experimental Details

MATERIALS AND METHODS

Construction of Plasmids: An *Eco* RI-*Bam* HI fragment containing the rtTA open reading frame was isolated from pHUD 17-1neo (Gossen et al., 1995). This fragment was cloned directionally into the mammalian expression vector pCDEF3 (Goldman et al., 1996) at the 5' *Eco* RI and 3'*Xba* I sites of the vector multiple cloning site to generate the final construct termed, EF1prtTA. Ligation of the 3'*Xba* I site of pCDEF3 and the BamHI site of the fragment was possible after Klenow filling the overhangs to make them blunt-ended. This

modified vector places the rtTA gene under direct transcriptional control of the human polypeptide chain elongation factor-1alpha promoter (EF-1a). Plasmids expressing the Mda-7 and Jun B cDNAs were constructed in pUHD 10-3 (Gossen and Bujard, 1992) by blunt cloning of isolated cDNA fragments into Klenow filled blunt vector followed by sequence analysis for confirmation.

Cell culture and derivation of stable cell lines: All cell lines used in this study were grown and maintained under standard conditions as previously described (Giang et al., 1996). Selection of stable clones expressing the rtTA cDNA using EF1prtTA was carried out in the presence of 500 to 1000 µg/ml G148 (Life Technologies Inc.) depending on the individual cell line. After the selection period, macroscopic visible colonies were picked, expanded and analyzed for activity by assaying for luciferase activity for rtTA expression or by Northern blot analysis of inducible cDNA such as Mda-7 or Jun B respectively.

Northern blotting: Total cellular RNA was resolved by denaturing formaldehyde agarose gel electrophoresis after isolation of RNA using an RNAeasy Kit (Qiagen). Transfer was done onto Hybond nylon membranes (Amersham) and probed with appropriately labeled cDNA probes for Mda-7 and Jun B.

Luciferase activity analysis: Luciferase assays were performed using a Luciferase Assay Kit (Promega) and quantitation was performed on a Turner Design TD 20/20 luminometer. Equal quantities of RNA were loaded on each gel following spectrophotometric estimation at 260 nm. Normalization of RNA levels between samples was confirmed by visualizing RNA on ethidium bromide stained gels. Normalization of luciferase activity was achieved by quantitating protein and adjusting the amount of extract to a fixed amount of protein.

RESULTS

Construction and initial testing of the EF-1a promoter based rtTA expression vector

Details of the cloning steps performed in construction of the EF-1 a promoter rtTA (EF1prtTA) expression vector is described in materials and methods and Fig 1. The protein expressed by this cDNA, a mutant form of the original bacterial Tc-repressor (Gossen et al., 1995), binds to and activates transcription of genes downstream of Tc-operator binding elements, only when Tc is present. EF1prtTA was transiently co-transfected with the Tc-responsive luciferase reporter plasmid, pUHC 13-3 (Gossen et al., 1995), into HO-1 human melanoma cells to determine if the construct was active. A parallel set of transfections was performed with the original CMV IE based construct, pUHD 17-1neo (Gossen et al., 1995) in the absence or presence of 1 µg/ml doxycycline(Dox). Cells were harvested 48 h after transfection and luciferase activity (Fig. 2) was determined using a luminometric Luciferase assay system (Promega). As previously documented (Gossen et al., 1994; Gossen and Bujard, 1992; Gossen et al., 1995) transient assays poorly reflect the level of inducibility actually obtainable after final selection of stable clones, since basal levels of expression change dramatically once plasmid DNA is integrated into chromatin. The initial experiments clearly demonstrated that the EF1prtTA expression vector was functional at comparable levels to the original pUHD 17-1neo construct in transient assays. Based on the positive activity obtained, the EF1prtTA construct was utilized to establish stable lines expressing rtTA in HeLa (human cervical carcinoma), HO-1, (human melanoma) MCF-7 (human breast carcinoma) and PC3 and DU-145 (human prostate carcinoma) cancer cell lines.

Analysis of stable cell lines expressing the rtTA cDNA under regulation of the EF-1α promoter

Cells were transfected with the EF1prtTA construct using Superfect transfection reagent (Qiagen) based on standard conditions recommended in the usage protocol. The efficiency of transfection, reflected by the number of clones obtained at the end of the selection period, varied with each cell line. Colonies were selected using Neomycin resistance conferred by the marker present within the construct. For every cell line, twenty-four Neomycin resistant colonies were isolated for further analysis. These individually selected clones were transiently transfected with the Tc-responsive luciferase reporter pUHC 13-3 (Gossen et al., 1995) to determine the presence and level of rtTA activity. Some cell lines used in this series of experiments had failed to generate Tc-responsive clones in previous attempts utilizing the CMV IE based construct pUHD 17-1neo (Gossen et al., 1995).

Results obtained in a screen to identify Tc-responsive clonal cell lines in HO-1, MCF-7, PC3 and DU-145 cells (Fig. 3) indicated that an average of at least two clones of the twenty-four or less clones finally analyzed per cell line, showed some levels of Tc-responsiveness. This frequency of positive clones is comparable, if not higher than that reported previously (Gossen and Bujard, 1992; Gossen et al., 1995). As mentioned above, the fold induction observed in the presence of Tc, though relatively low, is likely to be a reflection of leaky expression in uninduced conditions due to the transient transfection conditions used in this initial screen. Despite this leakyness, clones with high or low relative levels of inducibility were identifiable in every case and potentially usable cell lines were identfied with relative ease.

Functional analysis of stable clones expressing cDNAs under inducible regulation of EF1prtTA

In continuance of a major focus of our research involving analysis of the role of specific genes in melanoma differentiation, stable cells expressing differentiation associated genes including the transcription factor Jun B and the tumor suppressor Mda-7 (Jiang et al., 1996) under Tc-regulation were established in HO-1 melanoma cells. This human melanoma cell line has the ability to terminally differentiate in the combined presence of β-interferon and the Protein Kinase C (PKC) activator, mezerein. It is sensitive to culture conditions due to its capacity to differentiate, difficult to transfect and takes a relatively long time during selection to form visible colonies suitable for re-isolation as a clonal population of cells. HO-1 therefore presents an ideal proving ground for the efficacy of the EF-1 α promoter based vector. Using a suitable rtTA expressing cell line identified in the previous screen described above (Fig. 3), transfections were performed with the potentially Tc-regulatable Jun B and Mda-7 cDNAs cloned into the vector pUHD 10-3 (Gossen and Bujard, 1992). Colonies were isolated and individual clones were analyzed for expression and induction of Jun B and Mda-7 by Northern blotting. To determine the level of inducibility of individual clones, RNAs were isolated from each clone grown in the absence or presence of Dox. Northern blots, probed with Jun B and Mda-7 cDNA probes (Fig. 4) indicated that several positive clones had been obtained for each cDNA. As anticipated, varying degrees of clone dependent basal and inducible levels of expression was observed. It may be noted that the parental EF1prtTA cell line chosen from the initial screen (previous section, Fig 3) had not exhibited a very high level of fold inducibility in transient assays. However, on introduction of a Tc-operator regulatable construct, in a stably integrated form, high levels of Tc-dependent

induction was observed in individual clones (Fig. 4A compare lanes 1, 3, 9, 10 and 17, induced and uninduced level and similarly Fig. 4B lanes 1, 4 and 9). Overall, the frequency, variability and basal to induced levels obtained in various clones closely parallels thatreported for the Tc-system (Gossen et al., 1994; Gossen and Bujard, 1992; Gossen et al., 1995).

DISCUSSION

Inability to support continual strong expression from a given type of promoter, specifically those of viral origin has been documented for certain cell types (Gorman et al., 1985; Hasegawa et al., 1990 Li et al., 1992; Miller and Rizzino, 1995; Sleigh, 1987). The primary goal of this work is to reduce a significant and hitherto unaddressed variable in successfully establishing Tc-inducible cells. Expression of the Tc-operator expression construct, pUHD 10-3 (Gossen and Bujard, 1992) or its derivatives, into which the cDNA of interest is usually cloned, is ultimately dependent on expression of the tTA or rtTA gene product. Preventing or avoiding TA cDNA expression is shut down, during or subsequent to establishing a cell line, a variable that is likely to be cell type associated (Ackland-Berglund and Leib, 1995; Gossen and Bujard, 1995) should considerably enhance success rates. To achieve steady and adequate levels of the TA cDNA expression, relatively independent of temporal factors, cell-type, cell physiology status and cell passage number, we replaced the CMV IE promoter enhancer with the cellular EF-1α promoter (Goldman et al., 1996; Kim et al., 1990; Wakabayashi-Ito and Nagata, 1994). Experience in using pUHD 17-1neo (Gossen et al., 1995) indicated that while activity and inducibility in transient assays using sensitive detection methods with luciferase reporters worked reasonably well, we failed to generate cells showing any level of activity of the gene of interest after clonal selection of

individual lines, despite presence of expression construct DNA, in the genome using Southern analysis (data not shown).

Numerous modifications of the basic Tc-regulatable system have been reported in the literature directed toward enhancing performance. Several alternative promoters have been utilized to drive expression of the TA cDNA. Many of these are based on the requirement for tissue or species specific expression in plants (Weinmann et al., 1994), , Drosophila (Bieschke et al., 1998) or mice,(Bohl et al., 1997; Dhawan et al., 1995; Faiss et al., 1997; Hennighausen et al.,, 1995; Hoffmann et al., 1997; Holwell et al., 1997; Li et al., 1992; Liang et al., 1996; Miller and Rizzino, 1995; Thompson and Myatt, 1997). Another modification of the TA expressing construct involves use of bi- or multi-cistronic plasmid constructs which drives expression, through oppositely oriented promoters, of both TA-cDNA and Tc-operator regulated cDNAs, mainly to circumvent two rounds of transfection of separate plasmids (Baron et al., 1995 Fussenegger et al.,1997; Liang et al., 1996; Schultze et al., 1996; Weinmann et al., 1994). However they are based on one or a combination of viral promoters with accompanying drawbacks mentioned above. Multi-cistronic single retroviral or combinations of two or more retroviruses expressing different components has also been constructed (Bohl et al., 1997; Hofmann et al., 1996; Kringstein et al., 1998; Paulus et al., 1996; Rossi et al., 1998). These overcome the barrier of gene delivery into cells but again expression is often based on viral promoter sequences, prone to possible shutdown in some cell types. The relatively complex steps involved in making a virus for a given cDNA of interest including the intricate cloning strategies due to large vector size and investment in time, somewhat offsets the advantages they present over classical DNA transfection approaches. Making retroviral vectors is presently restricted to a relatively small proportion of laboratories and safety

concerns impose limitations of use in several setups. Therefore, while these vectors hold considerable promise, the likelihood of a major shift over to their usage from widespread DNA transfection approaches may only be in the long term. The relevance of improved plasmid vectors is therefore still strong.

A generally applicable modification to the original TA-expression construct involved expression of both TA-cDNA and exogenous cDNA under regulation of Tc-operator sequences (Liang et al., 1996; Shockett et al., 1995). The rationale being that, exquisite regulation with very high inducibility could be built into a system when both the activator molecule and the regulatable gene of interest are under control of the same inducer through an autoregulatory loop. Unfortunately, it appears that the high levels of tTA protein produced as a result of induction results in toxic side effects in cells (Gallia and Khalili, 1998; Gossen and Bujard, 1992) most likely due to interference in cellular metabolism by the acid activation domain of the HSV, VP16 protein present in TA-proteins. This could be an additional reason why certain cell types apparently shut down expression of TA-cDNA after extended periods time. Alternatively, cells strongly expressing TA proteins might be at a selective disadvantage, particularly in cells with a long doubling time due to accumulation of toxic levels of TA protein. While we can only speculate about the true reason for the apparent loss of TA expression, it appears that switching over to the EF-1 α expression cassette is able to balance out and over come these problems.

The conclusion is based on observations over periods of time, extending to almost twelve months in the case of certain EF1prtTA cells lines such as those established in HO-1 melanoma. The parental HO-1 EF1prtTA cell line was made and initially analyzed over a period of time (> 60 days) before

being expanded and frozen for future use. These parental cells were used to establish inducible Jun B and Mda-7 expression (Fig 4, A and B) that showed functional levels of TA-expression and inducible properties after being thawed out several months and passage numbers subsequent to when the line had initially been established and frozen. This line and others (Fig. 3) continue to retain Tc-responsive properties and were all maintained in the absence of antibiotic selection, indicating that expression of the rtTA cDNA continued irrespective of lack of positive selective pressure, passage number and time elapsed between introduction and integration of the plasmid DNA and final usage. Overall, following modification of the expression construct for the rtTA cDNA we have demonstrated that it had enabled us to significantly enhance the likelihood of establishing cell lines that are Tc-regulatable. It appeared that positive clones were obtained at higher frequencies than previously reported and that consistent expression and clonal stability over an extended period of time was accomplished. Based on these observations we conclude that the modified EF1prtTA presents a useful reagent with broad applicability in establishing Tc-regulatable cells.

Generation of Transgenic mice expressing the rtTA cDNA under control of the EF-1α promoter

Experiments to obtain expression of the rtTA protein in all tissues of mice, utilizing transgenic technology, are presently in progress. The EF-1α gene and its promoter are ubiquitously expressed in all animal tissues and is therefore a suitable expression system to achieve this goal. A transgenic expression cassette, consisting of the human EF-1α gene promoter linked to the rtTA cDNA has already been constructed and functionally tested in rat, mouse and human cell lines (described in the literature as pEF1prtTA (Gopalkrishnan et al., Nuc. Acids Res. 27:4775-4782, 1999 and

references therein)}. Standard procedures in the generation of transgenic mouse lines was performed. In brief, microinjection of pEF1prtTA into pronuclei of fertilized mouse eggs was carried out and these were implanted into psuedo-pregnant female mice. These manipulations resulted in a final litter of three mice which were analyzed for presence of the transgene in genomic DNA, derived from tail-tip samples by genomic Southern blot analysis using a radioactively labeled rtTA cDNA probe. This analysis revealed that one of the three founder mice was positive for the transgene since it displayed an appropriate sized band as detected by autoradiography. This founder, a female, has been subsequently crossed with wild type male mice to generate F1 progeny. Analysis of tail-tip DNA from the F1 generation has permitted us to determine whether the founder possess the capacity to transmit the transgene. Southern blot analysis of tail-tip DNA from 15 F1 generation indicated that eight (8) mice were positive for the transgene, confirming that the original founder animal had the capacity to transmit the inserted gene. Subsequent to our successful generation of transgenic mouse lines, we are presently in a position to breed additional animals and begin extensive expression analysis of the transgene to determine level of expression. This will be carried out on F1 or later generation mice, while maintaining the original founder until we are certain that stable expressing lines can be generated from progeny for future use and distribution. These mouse lines can be used to generate mice that can inducibly express specific genes under regulation of tetracycline to study the in vivo effect of specific genes in animals or screen for anti-tumoral or other pharmacological effects of drugs or small molecules.

Construction of pEF1ptTA, an expression vector expressing tTA, tetracycline repressor under regulation of the EF-1α promoter for significantly increasing success in establishing stable cell lines with consistent expression.

An EcoRI-BamHI fragment containing the tTA open reading frame was isolated from pUHD 15-1. This fragment was cloned directionally into the mammalian expression vector pCDEF3 at the 5' EcoRI and 3' XbaI sites of the vector multiple cloning site to generate the final construct, termed EF1ptTA. Ligation of the 3' XbaI site of pCDEF3 and the Bam HI site of the fragment was possible after Klenow filling the overhangs to make them blunt-ended. This modified vector places the tTA gene under direct transcriptional control of the human EF-1α promoter. The construct was confirmed by restriction enzyme and DNA sequencing analysis. Functional testing in rodent and human cell lines is presently underway and will be performed essentially as described for pEF1prtTA. Compared to the earlier construct (pEF1prtTA) wherein gene expression is induced in the presence of the inducer (tetracycline or doxycycline), the present construct is active in the absence of tetracycline or doxycycline and gene expression is shutdown in the presence of these reagents. Both plasmids may be used in setting up inducible gene expression systems in cell lines or mice and the choice will be dependent on whether one desires to grow cells in the presence or absence of the chemical agent.

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What is claimed is:

1. A vector comprising:
   a) Protein Translation Peptide Elongation Factor-1 α promoter; and
   b) nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor-1 α promoter.

2. The vector of claim 1 , wherein the vector is a plasmid.

3. The vector of claim 1, wherein the vector is as set forth in figure 1.

4. A cell comprising the vector of claim 1.

5. The cell of claim 4, wherein the cell is from a cell line.

6. The cell of claim 5, wherein the cell line is HeLa (human cervix), HO-1 (human melanoma), MCF-7(human breast), PC3 (human prostate) or DU-145 (human prostate).

7. The cell of claim 4, which consistently expresses tetracycline repressor.

8. A cell comprised of Protein Translation Peptide Elongation Factor-1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor-1 α promoter.

9. An animal comprising the vector of claim 1.

10. The animal of claim 9, wherein the animal is a mouse.

11. A method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression comprising:

a) isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion.

b) generation of Protein Translation Peptide Elongation Factor-1 α promoter vector, by restriction enzyme digestion;

c) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1 α promoter vector by ligation of 5' EcoRI compatible restriction enzyme overhangs;

d) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1 α promoter vector by Klenow fragment meiadte blunt end generation of 3' Bam HI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3' XbaI end of Protein Translation Peptide Elongation Factor-1 α promoter vector; and

e) blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor-1 α promoter vector expressing reverse tetracycline controlled transactivator.

12. The method of claim 11, wherein the fragment of 11(a) is an *Eco* RI-*BAM* HI fragment.

13. The method of claim 11, wherein the mammmalian expression vector of 11(b) is pCDEF3.

14. The method of claim 11, wherein the cloning of 11(a) is at the 5' *Eco* RI and 3' *BAM* HI sites.

15. The method of claim 11, wherein the ligation of 11(c) is at the 5' *Eco* RI site of pCDEF3.

16. The method of claim 11, wherein the ligation of 11(d) is at the 3' XbaI site of pCDEF3.

17. A vector generated by the method of claim 11.

18. A method for screening pharmacological products using the vector of claim 1.

19. A method for monitering inducible gene expression in a tissue specific or generalized manner using the vector of claim 1.

Abstract of the Disclosure

The present invention provides a vector having Protein Translation Peptide Elongation Factor-1α promoter and nucleic acids encoding reverse tetracycline controlled activator, wherein the expression of said activator is under the control of Protein Translation Peptide Elongation Factor-1α promoter. In addition, the invention provides a method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression having: (a) isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion, (b) generation of Protein Translation Peptide Elongation Factor-1α promoter vector, by restriction enzyme digestion, (c) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1α promoter vector by ligation of EcoRI compatible restriction enzyme overhangs, (d) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1α promoter vector by Klenow fragment mediated blunt end generation of 3' BamHI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3' XbaI end of Protein Translation Peptide Elongation Factor-1α promoter vector and (e) blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor-1α promoter vector expressing reverse tetracycline controlled transactivator.

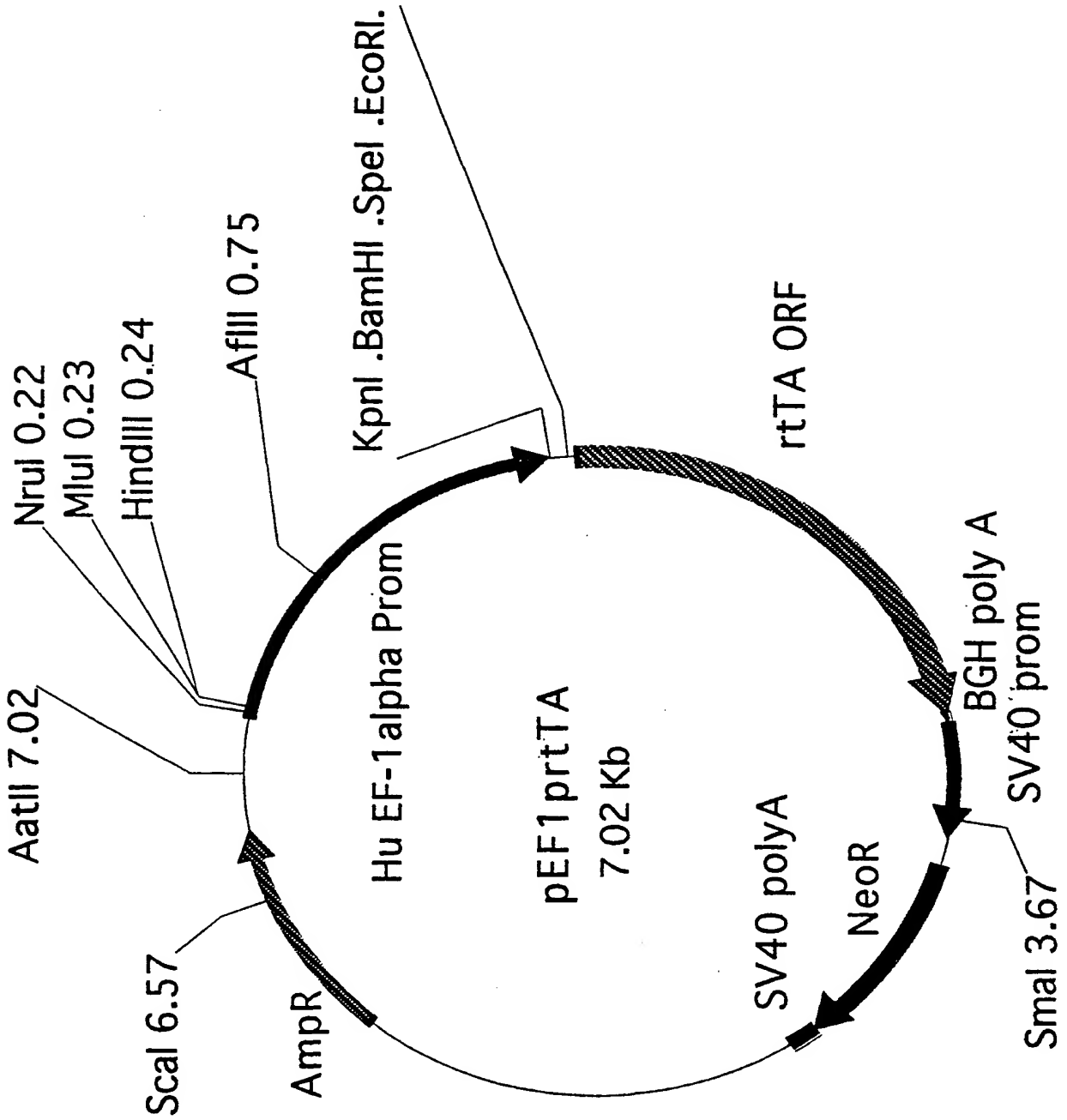

KpnI .BamHI .SpeI .EcoRI.
AflII 0.75
HindIII 0.24
MluI 0.23
NruI 0.22
AatII 7.02
ScaI 6.57
AmpR
Hu EF-1alpha Prom
pEF1prtTA
7.02 Kb
rtTA ORF
BGH poly A
SV40 prom
SmaI 3.67
NeoR
SV40 polyA

FIG. 1

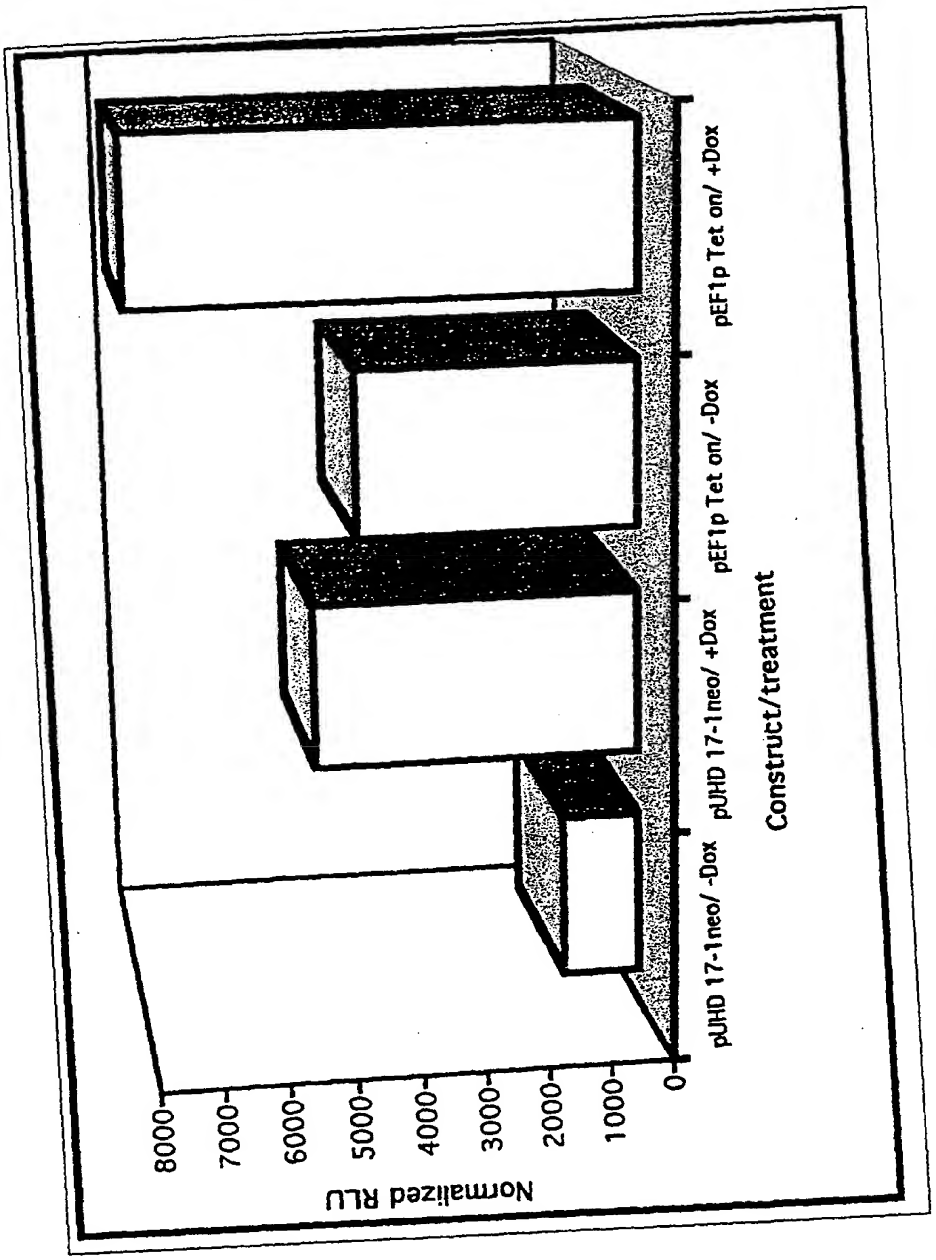

Normalized RLU
8000
7000
6000
5000
4000
3000
2000
1000
0
pUHD 17-1neo/ -Dox
pUHD 17-1neo/ +Dox
pEF1p Tet on/ -Dox
pEF1p Tet on/ +Dox
Construct/treatment

FIG. 2

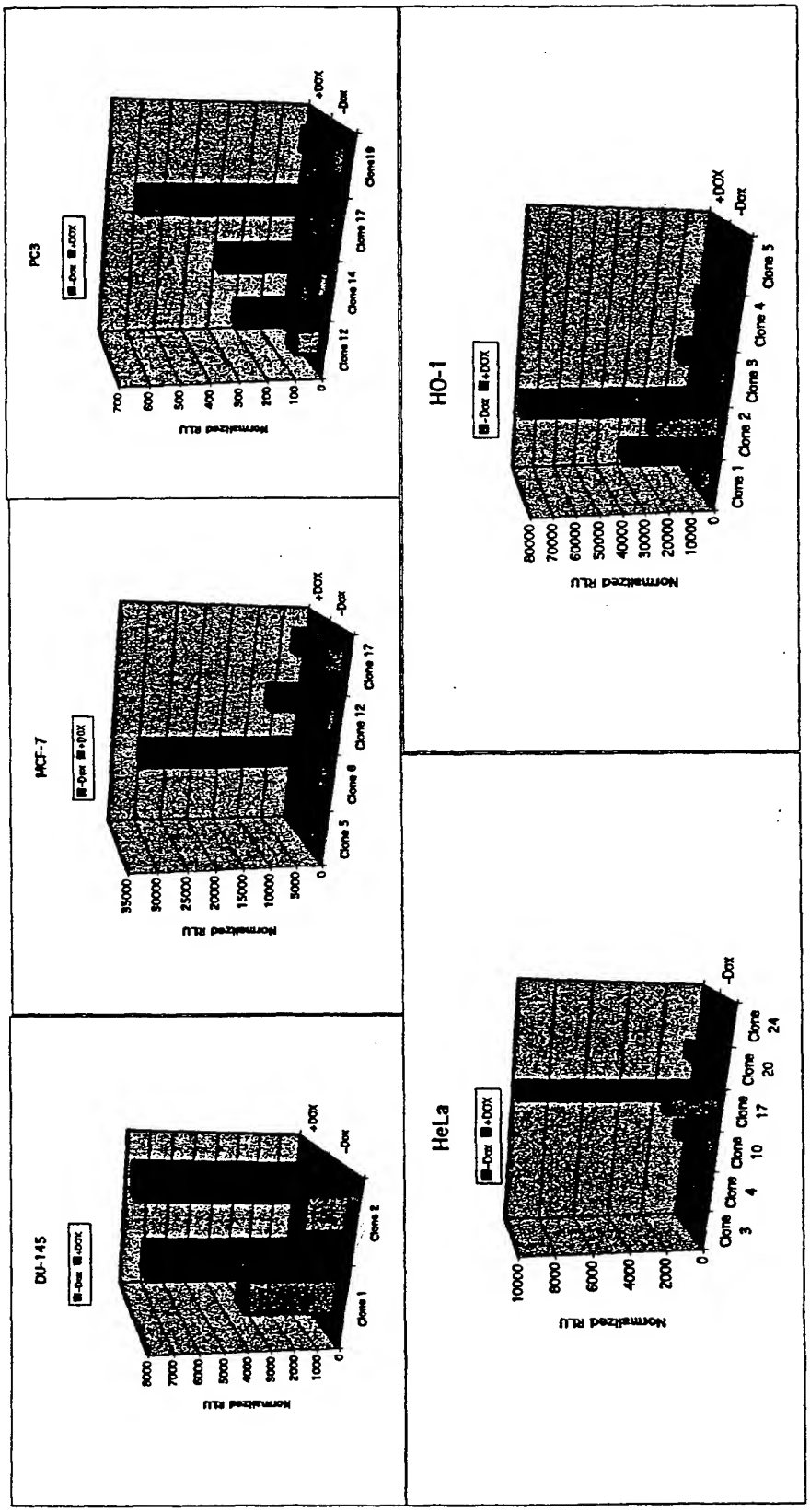


FIG. 3A
DU-145
-Dox
+DOX
Normalized RLU
Clone 1
Clone 2
FIG. 3B
MCF-7
-Dox
+DOX
Normalized RLU
Clone 5
Clone 8
Clone 12
Clone 17
FIG. 3C
PC3
-Dox
+DOX
Normalized RLU
Clone 12
Clone 14
Clone 17
Clone 19
FIG. 3D
HeLa
-Dox
+DOX
Normalized RLU
Clone 3
Clone 4
Clone 10
Clone 17
Clone 20
Clone 24
FIG. 3E
HO-1
-Dox
+DOX
Normalized RLU
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5

Figure 3

FIG. 4A

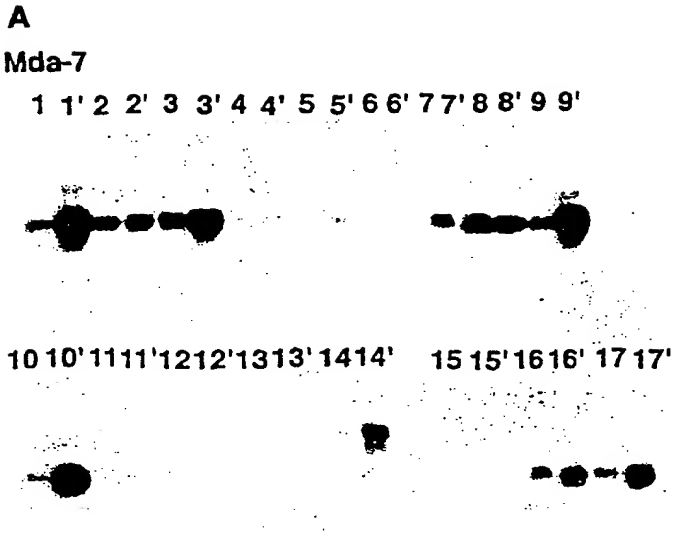


FIG. 4B

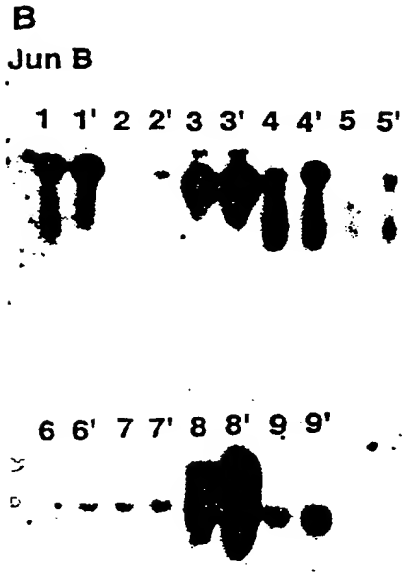
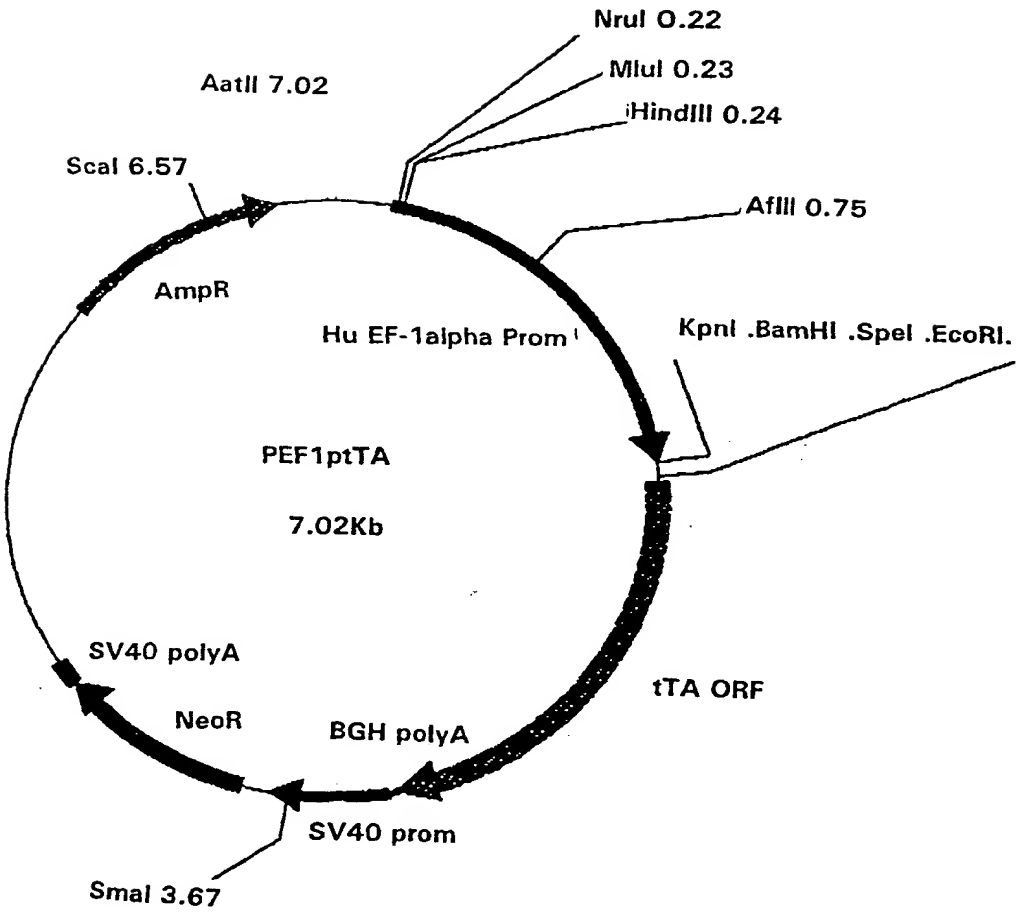


Figure 4

Figure 5


NruI 0.22
MluI 0.23
HindIII 0.24
AatII 7.02
ScaI 6.57
AflII 0.75
AmpR
Hu EF-1alpha Prom
KpnI .BamHI .SpeI .EcoRI.
PEF1ptTA
7.02Kb
SV40 polyA
tTA ORF
NeoR
BGH polyA
SV40 prom
SmaI 3.67

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/06862

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 15/00, 5/00, 15/63; C12P 21/06; C12Q 1/68; A01K 67/00
US CL :435/6, 320.1, 325, 69.1, 455; 800/13, 18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 320.1, 325, 69.1, 455; 800/13, 18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, BIOSIS, MEDLINE, CAPLUS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GOSSEN et al., Transcriptional Activation by Tetracyclines in Mammalian cells, Science, June 1995, Vol. 268, p. 1766-1769, entire document.	1-8, 11-17, 19
Y	GOLFMAN et al., Modifications of Vectors pEF-BOS, pcDNA1 and pcDNA3 Results in Improved Convenience and Expression, BioTechniques,December 1996, VOl. 21, No. 6, p. 1013-1015, entire document.	1-8, 11-17, 19
Y	US 5,654,168 A (BUJARD et al) 5 August 1997, especially column 20, 25, 33, 34, 49, 50.	1-19
A	PURSEL et al., Expression and Performance in Transgenic Pigs, J. Reprod. Fert., 1990, Suppl. Vol. 40, p. 235-245.	9, 10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 03 MAY 2000	Date of mailing of the international search report 19 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SHIN-LIN CHEN Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PALMITER et al., Metallothionein-Human GH Fusion Genes Stimulate Growth of Mice, Science, November 1983, Vol. 222, p. 809-814.	9, 10
A	KAPPEL et al., Regulating Gene Expression in Transgenic Animals, Current Opinion in Biotechnology,1992, Vol. 3, p. 548-553.	9, 10